THE EFFECTS OF THE AMINO ACIDS L ALANINE AND GLYCINE ON THE GERMINATION AND SURVIVAL OF SPORES OF BACILLUS MEGATERIUM

A Thesis

Presented to

The School of Graduate Studies

Drake University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts in Biology

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by

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September 1971

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INTRODUCTION AND REVIEW OF THE LITERATURE

The bacterial endospore has been a subject of much interest and research to biologists ever since it was observed and described by Perty in 1852. Pasteur, in 1870, confirmed the observations of Perty when he too found similar elliptical, light-refracting bodies in bacteria. He extended the knowledge concerning the properties of these bodies by observing that they possessed greater resistance to injurious agents than do bacteria cells themselves. Cohn, in 1877, was the first to establish the true nature of these bodies by observing the appearance of the light-refracting spores within the cell. He also observed the process of germination of spores of Bacillus subtilis directly with the microscope.

Since the earliest work, bacterial spores have been the basis of controversies and the subject of many areas of research. Numerous theories have evolved concerning the causes and conditions, both cellular and environmental, leading to spore formation. One of the first was that of Buchner's in 1890, which indicated that the exhaustion of nutrient materials as the primary factor. Preisz, in 1904, considered sporulation as a definite stage in the development of bacilli, and held that the optimum conditions for the initiation of the process were identical with those favoring maximum vegetative development. Matzuschita, in

1902, recognized lack of nutrient material as a factor, but attached more significance to oxygen accessibility in sporulation. Henrici (1924) showed that spore formation is initiated at the end of the active growth stage of a culture, and due to this fact, sporulation proceeds more rapidly in quarter strength medium because of the earlier cessation of active growth. Churchman (1925) attacked the concept that sporulation is merely a protective mechanism brought about by the stimulus of adversity in the environment and presented a certain amount of evidence from the standpoint of dye inhibition of sporulation in support of his contention. The complexity of the phenomenon of sporulation was further indicated in the work of Daranyi in 1927, who attributed the principal role to colloidal influences. His explanation was that upon aging a shrinking of the cell colloids occurs which brings about the stimulation necessary for the initiation of spore formation.

The more accepted theory today is that under certain conditions, which are not well understood, some species of bacteria form bodies within their cytoplasm capable of withstanding influences adverse to bacterial growth. These bodies are called spores. When conditions suitable for bacterial growth are established, the spore germinates back into the original actively multiplying bacterial form. The germinating spore becomes the vegetative form of the bacterium. Spore formation seems to be a characteristic

of bacilli, being exceedingly rare in cocci and spirilla. Among bacilli about one hundred fifty species form spores. For a time, it was thought that spore formation was a protective phenomenon which came into play when bacteria were grown under adverse conditions, but investigations have proved that this is not the case. Spore formation seems to be a normal phase in the life of certain species of bacteria. There is a temperature for each species at which spore formation is most active, and spore formation is preceded by a period of active vegetative reproduction. Some species form spores only in the presence of oxygen, and others, only in its absence. Some observers regard spore formation as a method of bringing about a rest, a kind of hibernation for the organism. Spore formation is not a reproductive phenomenon, according to Pelczar and Reid (1965).

Much information is now available to us concerning spores. The process of spore formation is as follows. When a certain period arrives within the life cycle of the bacteria, a hard, round, or roundish body forms within the cell so that the cell may begin to bulge at the center, subterminally, or at one end. Within the round body is the concentrated protoplasm of the cell. Eventually the rest of the vegetative cell wall breaks down and frees the mature endospore. This new kind of cell carried within it all the potentialities necessary to form another vegetative cell.

Endospores have remarkable physiological properties according to Sussman and Halvorson (1966). The outstanding one is their exceptional thermal resistance, much greater than that of any other known cell. Spores are also resistant to such adverse factors as heat, dessication, toxic chemicals and ionizing radiation. They do not stain with simple aqueous dyes as methylene blue; they are refractile; they contain two to ten times as much calcium as vegetative cells; and from five to fifteen per cent of their dry weight is dipicolinic acid. DPA may be concerned with heat resistance and with low metabolic activity of spores. It is not found in germinated spores nor in vegetative cells.

The conditions for growth and sporulation are generally similar, nevertheless, the limits are more restricting for the latter. The physical environment of the cell is influenced by factors like temperature, pH, oxygen requirements, water activity, surface tension, and others. Temperature has been found to affect the rate and the amount of spore formation. Some biochemical processes involved in sporogenesis may also be affected by temperature. In 1931, Christian observed that a species of Bacillus formed "abortive spores" at the maximum temperature for spore formation. However, they were not viable.

Some species have an optimal pH of 5.4 to 5.5 for sporulation (Bowen and Smith, 1955), whereas others have a higher optimal pH range. The change in the pH of a culture

is an expression of the change in its metabolism which accompanies growth and sporulation. Halvorson (1957) observed that the initial drop in pH (from 7.0 to 5.5) closely followed the first peak in oxygen demand of B. cereus strain T. The pH began to rise again concomitantly with the second peak in the oxygen demand curve.

Recent investigations on the requirements of the aerobic spore former indicate that relatively more oxygen is required for sporulation than for vegetative growth. B. anthracis and B. globigii required higher aeration rates for complete sporulation than for maximum growth (Roth, Lively and Hodge, 1955). The oxygen demand reached a peak where cells of B. cereus strain T attained a maximum population and the glucose was exhausted, but in instances where the first spores formed (within 9 hrs.), the oxygen demand fell (Halvorson, 1957).

Just as researchers have been interested in the process of sporulation, equal interest has been shown in the process of germination of the bacterial spore. Much time and space has been given to defining this germinating process. One of the first, De Bary (1887) said germination consists chiefly in the development of the spore into a cell which assumes all the characters of the parent-cell as regards conformation and vegetation. Some of the more recent investigators agree with this definition. These include Fitz-James and Knaysi, among others. Still other authorities apply

physiological criteria to judge the end of germination. Among these physiological changes that occur after the bacterial spore is activated are the following: loss of resistance to environmental stresses, such as extremes of temperature, dessication, radiation, and chemicals; loss of refractility; increased stain ability; reduction in optical density of spores in water; swelling of the spore; and others. Most of these changes have been used as indexes of germination; however, Knaysi (1948) criticizes this method and prefers to use the appearance of the vegetative cell as the criterion.

Spore germination in bacteria may be regarded as the change from a heat-resistant spore to a heat-labile entity which may not necessarily be a true vegetative cell. This definition was provided by Campbell (1957), and has the advantage of a convenient and an accurate measurement, because the degree of heat resistance is an important and apparently universal difference between spores and vegetative cells of bacteria. There also seems to be a good correlation between the change in heat-resistance and other physiological changes that occur once the spore is activated.

Sussman and Halvorson (1966) defines germination by saying it "occurs when the first irreversible stage that is recognizably different from the dormant organism is reached, as judged by physiological or morphological criteria." In arriving at this definition, guide lines for the evaluation

of germination are as important as the definition. The marker for judging must be a stable and ubiquitous one that commits the spore to further development; the stage reached should be irreversible.

In recent years there has been an increased interest in the understanding of the processes leading to the termination of the cryptobiotic state in the bacterial endospore and the transformation of the spore into a vegetative cell. Although the exact mechanisms are not understood, a generalized picture of the events occurring in the spore during its transformation into a vegetative cell has begun to emerge. On the basis of present knowledge, three different kinds of sequential events occur. According to Keynan and Halvorson (1965), these are called activation, germination, and outgrowth. Activation is a process which modifies the spore to germinate under appropriate circumstances. Evidence indicates this process is reversible. Activation results in a spore which is ready for germination, but retains most spore properties. The second process, germination, is irreversible, and results in a cell which has lost the typical characteristics of a bacterial spore. Germination is a process of degradation of macromolecules, resulting in the excretion of many typical spore substances into the medium. The third process, outgrowth, is a process of synthesis of new macromolecules which result in the emergence of a new vegetative cell.

Activation may also be referred to as the breaking of dormancy of the endospore. Many freshly prepared spore suspensions will not germinate under a given germination condition unless heated or aged. The recognition that the dormant state could be broken by the application of heat was first clearly recognized by Curran and Evans (1945), and is usually called "heat shock" or "heat activation." The heat-activated spore does not lose its characteristic spore properties, but changes its qualitative and quantitative requirements for the induction of germination. It has long been known that less exacting conditions will induce and enhance germination in heat-activated spores (Powell and Hunter, 1955). Some spore suspensions will germinate without activation; others need temperatures up to 105 degrees Centigrade to be activated. This phenomenon can best be explained as a process of a reversible structural change in macromolecules. A study of heat activation shows that the activation energy is high, similar to that of heat-denaturation of macromolecules (Busta and Ordal, 1964). Also, activation can be imitated chemically by reducing agents and low pH (Keynan, Evenchik, Halvorson, and Hastings, 1964) which denatures proteins.

Whatever notion we have of the reactions involved in the induction of germination, the outcome of every kind of germination described, physiological, chemical, or mechanical, is always the irreversible loss of a typical pattern of

spore properties. The resulting cell is metabolically active, heat-labile, nonrefractile, stainable, and yet readily distinct from vegetative bacteria. Wynne (1952) was among the first to point out that different conditions are necessary for growth and germination, and therefore these are two different processes. Levinson and Hyatt (1956) observed that special nutritional requirements are necessary to convert the spore into a vegetative cell.

Conditions for outgrowth are usually different from those supporting germination. The two processes have a different temperature optimum, and most spores need nutrients for outgrowth which are not needed for germination. Outgrowth is sometimes divided into two areas, post-germination and prevegetative (Sussman and Halvorson, 1966). This process includes increase in respiratory capacity, loss of brilliance under dark-contrast phase-microscopy, and loss of dipicolinic acid (DPA) and heat resistance. At this time the spores become stainable and may increase in size.

A study undertaken by H. A. Thompson at Drake University investigated the effects of ultra violet light upon the spores of Bacillus subtillis immersed in low concentrations of amino acids. The amino acids used were L - histidine and DL - leucine. The results of this investigation showed that L - histidine had no significant effect upon the number of irradiated spores which germinated but may have an effect upon non-irradiated spores. The results with DL - leucine

showed this acid did affect the number of spores that germinated.

This study was concerned with a comparative study of the effects of two amino acids, alanine and glycine on the germination of spores of Bacillus megaterium. Alanine and glycine were selected because both of these acids are used in metabolism. Also they have relatively simple structure and are water soluble.

MATERIALS AND METHODS

Bacillus megaterium, the bacterial organism used in this study, was obtained from a stock culture maintained at Drake University, Des Moines, Iowa. The organism was cultured on nutrient agar and held at a temperature of five degrees Centigrade until subcultures were needed. New subcultures were prepared monthly throughout the course of this investigation.

Spores were produced by inoculating culture tubes containing nutrient agar with Bacillus megaterium. These tubes were incubated at thirty-seven degrees Centigrade for ten days. At the end of this time the spores were harvested and held in ten milliliters of sterile distilled water at five degrees Centigrade until ready to be used.

All growth media used in this investigation was obtained from Difco Laboratories, Incorporated, (Detroit, Michigan). Difco nutrient agar was used to start subcultures

and to culture spores. Difco tryptic soy broth, alanine and glycine (Difco), were used in preparing the different media to test germination of the spores of Bacillus megaterium.

All materials and glassware used in this study were sterilized in a Castle #999C Autoclave (Fisher Scientific Company, St. Louis, Missouri), at fifteen pounds pressure for twenty minutes. All glassware was washed with Laboratory Cleaner (Carolina Biological Supply Company, Burlington, North Carolina), rinsed in tap water, and distilled water before sterilization. Standard pyrex culture tubes, size 20mm x 150mm, and stoppers of cotton plugs were used throughout the investigation. Cultures were incubated in a Cenco incubator (Central Scientific Division of Cenco Instruments Corporation, Chicago, Illinois).

Spores were harvested from ten day old nutrient agar cultures in the following way. An inoculating needle was bent into an "L" shaped tool. This needle was sterilized before using it to scrape the surface of the agar in removing the growth from the agar. This crop was then suspended in sterile culture tubes containing ten milliliters of sterile distilled water and containing glass beads (three millimeters in diameter). This mixture was shaken vigorously for ten minutes and then stored at five degrees Centigrade. This was the stock culture of spores for the determination of spore germination.

The capacity of the inoculating loop to be used in the investigation was determined. This was accomplished by taking a known volume and counting the number of loops that could be obtained from this volume. The number of loopfuls divided into the known volume would give the capacity of one loopful. It was also helpful to have a determined area on a slide so that a smear could be contained. Representative areas could be counted and these could be adjusted to take in the entire area on the slide. The method of establishing the area on a slide was to scratch an eight millimeter square. This area was then subdivided into two millimeter squares. A jeweler's file was used to scratch these areas on the slide.

The microscope used throughout the investigation was a Cenco Research Microscope (Central Scientific Company, Chicago, Illinois) equipped with a Bausch and Lomb ocular micrometer and a graduated mechanical stage.

The amino acids used were L-alanine and glycine obtained from Difco Laboratories (Detroit, Michigan). The different media was prepared by weighing appropriate amounts of tryptic soy broth and the amino acids on a Volland 100 Analytical Balance (Volland Corporation, New Rochelle, New York). The concentration of the solutions used was determined by reviewing the literature pertinent to this investigation. Levinson and Hyatt (1956) used a 0.2mM concentration of L-alanine in their study of the effect of this amino acid on spores of Bacillus megaterium. Later work of Levinson and

Hyatt (1963) show that concentrations of 25 mM of alanine were used. For the purpose of this study the concentration of alanine used was between these two extremes at 10 mM. Preliminary studies indicated that low concentrations of alanine seemed to influence germination of spores. A 10 mM concentration of amino acids was arbitrarily selected for this investigation.

In order to investigate the effect of the amino acids alanine and glycine on the germination of spores of Bacillus megaterium four different media were used in each group of experiments. The different media were: Medium A, tryptic soy broth; Medium B, tryptic soy broth with a known amount of L-alanine; Medium C, tryptic soy broth with a known amount of glycine; and Medium D, tryptic soy broth with equal amounts of L-alanine and glycine. A known number of spores was added to each of the four media. These were then heat-shocked. This was accomplished by placing the tubes in a water bath at sixty degrees Centigrade for thirty minutes. After this, the spore cultures were incubated at thirty-seven degrees Centigrade. During this incubation simple stains from smears were made every hour for five hours (Levinson and Hyatt, 1956). Since germinating spores stain with methylene blue while ungerminated spores do not, this stain was used in this investigation.

Medium A, which served as the control as well as the basic components for the other two media, was prepared in

the following way. Thirty grams of powdered tryptic soy broth was dissolved in one liter of distilled water. Ten milliliters of this solution was dispensed into standard culture tubes, plugged with cotton, and sterilized at fifteen pounds pressure for twenty minutes. It was then refrigerated at five degrees Centigrade until needed.

Medium B was prepared by making a solution of tryptic soy broth as described above and alanine added to produce the desired molarity, 10.0 mM, of alanine. Ten milliliters of this solution was dispensed into standard culture tubes, cotton plugged, sterilized at fifteen pounds pressure for twenty minutes, and refrigerated at five degrees Centigrade until needed. Amino acids are non-volatile crystalline substances which do not decompose except at fairly high temperatures. Therefore the alanine and glycine were added to the minimal broth prior to sterilization.

Medium C was prepared in a similar way by substituting glycine for alanine. A solution of tryptic soy broth was prepared as previously described and powdered glycine added to produce a 10.0 mM concentration of the amino acid. Ten milliliters of this solution was dispensed into standard culture tubes, cotton plugged, sterilized at fifteen pounds pressure for twenty minutes, and refrigerated at five degrees Centigrade until needed.

Medium D was prepared by making a solution of tryptic soy broth and adding equal volumes of L-alanine and glycine

to produce 10 mM concentration. Ten milliliters of this solution was dispensed into standard culture tubes, cotton plugged, sterilized at fifteen pounds pressure for twenty minutes, and refrigerated at five degrees Centigrade until needed.

Prior to testing the effect which the amino acids would have on the germination of the spores of Bacillus megaterium, the concentration of the spore inoculum was determined. This was accomplished by charging an inoculating loop, whose volume had been determined earlier, with the spore suspension. This inoculum was then placed on the slide within a definite area determined earlier. The smear was stained with methylene blue dye and the number of spores determined by counting twenty-five different fields. The average number of spores per field was calculated from these twenty-five fields. Since the area of a single field was known, and the number of these fields per area occupied by the spores also was known, the number of spores in a given volume could be determined. This procedure was repeated ten times in order to obtain the average number of spores.

The actual experiments were carried out in series containing the four media described earlier. In each series the germination of spores in Medium A, Medium B, Medium C, and Medium D was determined. In any given series four culture tubes were used, one each with Medium A, Medium B, Medium C, and Medium D. All were inoculated with one

milliliter of the known spore inoculum. This transfer was accomplished by use of a sterile one milliliter pipette. These culture tubes were then placed in a water bath at sixty degrees Centigrade for thirty minutes. This was done for two reasons: first, heating of spores seems to be a triggering agent in inducing spores to germinate; and second, holding a temperature of sixty degrees Centigrade for thirty minutes would kill vegetative cells that might be present at this time. After the heat-shock treatment the cultures were placed in an incubator at thirty-seven degrees Centigrade and held for five hours.

The cultures were examined at the end of each hour for five hours to determine the percentage germination of the spores. One of the more useful ways of determining the germination of spore suspensions is that the suspension becomes less turbid and previously nonstaining spores become stainable with methylene blue. Ideally, turbidity as a measure of spore concentration could be determined with a spectrophotometer which has the advantage of being rapid, reproducible, and nondestructive (Koch and Crandall, 1968). Since a spectrophotometer was not available in my laboratory, the alternative method described was used. The percentage germination of spores was determined by dividing the number of vegetative cells stained with methylene blue by the total number of spores present at zero hours. By determining this percentage, the effect of the amino acids

L-alanine and glycine on the germination of spores of Bacillus megaterium could be ascertained.

DATA AND DISCUSSION

Spores of Bacillus megaterium, grown on nutrient agar, harvested, and kept in distilled water at five degrees Centigrade were used in this investigation. Known volumes of this harvest were inoculated into Medium A which contained tryptic soy broth and served as the control. Other spores were inoculated into one of three media: Medium B, containing tryptic soy broth and the amino acid L-alanine in a concentration of 10 mM; Medium C, containing tryptic soy broth and the amino acid glycine in a concentration of 10 mM; Medium D, containing tryptic soy broth and both amino acids L-alanine and glycine.

Spores were inoculated into the culture medium which was then placed in a water bath held at sixty degrees Centigrade. Cultures were kept in this water bath for thirty minutes. At the end of this period cultures were incubated at thirty-seven degrees Centigrade. Hourly checks were made to determine the rate of germination.

Preliminary checks on the rate of germination of spores of Bacillus megaterium showed that very little germination occurred during the first hour of incubation. Further investigation indicated that most spores would germinate within a five hour period. Therefore, for the

purpose of this study, counts were made at the end of two hours and three hours incubation.

Data was collected by counting the number of vegetative cells and the number of spores in each of the four media after two hours and three hours incubation. From these counts the per cent germination could be calculated. These counts and the calculated per cent germination are shown in Tables I through IV. The mean for the germination in each media is also shown in Tables I through IV.

In the analysis of the data obtained, it was of importance to determine whether a difference occurred between the germination of spores grown in the control Medium A, and Medium B, Medium C, and Medium D. If there was a difference then it becomes of importance to determine the degree of difference, and, if possible, to ascertain an explanation for such growth. The mean for the per cent germination for each medium was determined and a student's "t" test was made comparing Medium A to Medium B, Medium C, and Medium D. Differences were taken as significant if the "t" value was above 2.43 for a ninety-five per cent level of confidence. The "t" values and significance for the germination studies are shown in Table V for the results of two hours of incubation. Table VI shows the results for three hours of incubation.

The results obtained showed that small variations occurred between the control group of spores grown in tryptic

TABLE I. Germination of *Bacillus megaterium* spores after two hour incubation in medium A and medium B

Test	Medium A	Per cent Germination	Medium B	Per cent Germination
1	124	40	116	53
2	127	45	108	50
3	84	30	128	40
4	95	30	182	45
5	124	40	195	50
6	104	38	208	52
7	51	25	100	40
8	54	30	93	35
9	115	30	80	40
10	140	43	145	50
11	95	47	190	50
12	75	25	245	42
13	188	45	187	47
14	228	43	236	45
15	158	35	221	45
16	105	28	113	39
17	98	31	208	47
18	104	38	180	44
19	198	39	210	43
20	115	31	110	34
MEAN		35.65		44.55

TABLE II. Germination of Bacillus megaterium spores after two hour incubation in medium C and medium D

Test	Medium C	Per cent Germination	Medium D	Per Cent Germination
1	116	42	83	47
2	81	48	118	51
3	97	33	180	45
4	92	40	179	47
5	81	30	185	45
6	103	35	160	40
7	78	33	84	30
8	70	37	124	40
9	85	25	100	35
10	80	37	140	45
11	160	45	145	50
12	100	35	115	40
13	140	38	182	45
14	200	40	223	48
15	122	30	176	37
16	125	33	105	40
17	82	29	233	44
18	103	34	127	43
19	135	33	239	45
20	90	30	142	35
MEAN		35.35		42.60

Legend

Medium A - Tryptic soy broth

Medium B - Tryptic soy broth with L-alanine

Medium C - Tryptic soy broth with glycine

Medium D - Tryptic soy broth with L-alanine and glycine

TABLE III. Germination of *Bacillus megaterium* spores after three hour incubation in medium A and medium B

Test	Medium A	Per Cent Germination	Medium B	Per Cent Germination
1	270	70	313	78
2	188	65	243	75
3	340	83	363	85
4	328	80	419	83
5	274	76	356	75
6	285	75	333	70
7	126	60	214	65
8	150	65	156	65
9	175	70	214	65
10	140	70	300	75
11	262	68	299	73
12	245	70	293	75
13	396	80	382	81
14	240	80	220	80
15	301	85	325	81
16	238	70	276	85
17	472	80	500	80
18	250	72	362	80
19	310	73	295	82
20	379	74	392	80
MEAN		73.30		76.65

TABLE IV. Germination of Bacillus megaterium spores after three hour incubation in medium C and medium D

Test	Medium C	Per Cent Germination	Medium D	Per Cent Germination
1	261	75	296	80
2	210	70	323	77
3	304	80	357	85
4	312	80	510	85
5	228	65	392	80
6	258	68	329	70
7	119	70	330	75
8	135	60	214	60
9	190	68	260	70
10	180	65	145	65
11	210	70	285	75
12	238	70	301	75
13	334	81	406	83
14	241	81	246	78
15	294	70	312	80
16	300	84	265	85
17	404	77	484	83
18	405	75	381	80
19	217	70	326	80
20	184	68	285	78
MEAN		72.35		77.20

Legend

Medium A - Tryptic soy broth

Medium B - Tryptic soy broth with L-alanine

Medium C - Tryptic soy broth with glycine

Medium D - Tryptic soy broth with L-alanine and glycine

TABLE V. "t" values for spore cultures of Bacillus megaterium grown in the presence of L-alanine and glycine for two hours compared to the control

	Group	"t" value	Significance
B	L-alanine	4.49	+
C	glycine	.147	-
D	L-alanine and glycine	3.515	+

"t" for 95% confidence level is 2.43.

TABLE VI. "t" values for spore cultures of Bacillus megaterium grown in the presence of L-alanine and glycine for three hours compared to the control

	Group	"t" value	Significance
B	L-alanine	1.63	-
C	glycine	.46	-
D	L-alanine and glycine	1.85	-

"t" for 95% confidence level is 2.43.

soy broth and the test group with alanine, glycine, or both alanine and glycine. The greatest difference at the two hour incubation period was registered between the control group and the group referred to as Medium B which contained the amino acid L-alanine. The smallest degree of spore germination occurred in the medium containing glycine.

In the results for the third hour of incubation a smaller individual difference was shown in spore germination for Media B, C, and D. Again, the greatest difference is registered with the medium containing alanine. This difference, however, is more noticeable between the D culture containing both amino acids and the C culture containing glycine.

It would appear that when alanine was added to the basic medium, it seems to influence greater germination of spores at the second hour of incubation than it does at the third hour of incubation. Germination of spores in the glycine treated broth was not as great as in the control broth at the end of three hours of incubation. No reports were found in the literature that glycine had an inhibitory effect on germination, nor accelerates germination.

SUMMARY AND CONCLUSIONS

When spores of Bacillus megaterium were inoculated into a liquid medium, there was a short interval between their germination and subsequent development into a culture of growing cells. Opinions concerning the mechanism of

germination are divergent. However, the statement that spore germination may be considered as a change from a heat resistant state to a heat labile entity is generally descriptive of the process. Stainability and a decrease in optical density of the spore suspension may be used as criteria for germination.

This investigation has attempted to measure the influence on germination of spores of Bacillus megaterium by adding limited quantities of the amino acids L-alanine and glycine to tryptic soy broth. Spores were added to each of four media: tryptic soy broth; tryptic soy broth with alanine; tryptic soy broth with glycine; tryptic soy broth with alanine and glycine. These cultures were heat-shocked and then incubated for a limited period of time. At an interval of two hours slides were made, vegetative cells were counted and the per cent germination calculated. This was repeated at a three hour interval. It was repeated again at a four hour interval but germination was so complete that this interval was disregarded.

The data obtained during the investigation indicated that alanine has a significant effect on the germination of the spores. This seems to agree with most of the literature that states that alanine has an accelerating effect on spore germination. The mixture of tryptic soy broth with alanine, and the mixture of tryptic soy broth with alanine and glycine showed the most rapid and uniform darkening of the spores,

as well as the greatest number of vegetative cells. An important fact not discernible from the table is that spores germinating in an alanine enriched medium begin germinating before those spores introduced into the control and glycine media. Spores germinating in the control and glycine media were slower in germination but reached their maximum in two hundred forty minutes to three hundred minutes.

The results obtained are typical of those obtained in twenty series of experiments. It would seem that various physical and chemical factors may serve as activators for the germination and growth of spores of cultures of bacteria. One of the most frequently mentioned activators in the germination process is heat. In this experiment spores were heat-shocked which seemed to stimulate the germinating process. Certain chemicals seem to initiate germination of bacterial spores. The results of this study would indicate that of the two amino acids tested, alanine had a definite effect on the germination of the spores of this strain of Bacillus megaterium. The effect of glycine was less noticeable as the rate of germination in the glycine enriched media was the same as the tryptic soy broth.

Further studies might investigate the inhibitory effect of glycine on germination if larger quantities were used. Other studies might investigate the influence of alanine if added later in the germinating process of spores of Bacillus megaterium.

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